TISSUE ENGINEERED TESTICULAR PROSTHESES WITH PROLONGED TESTOSTERONE RELEASE

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ABSTRACT

Young soldiers with testicular tissue injury may require testicular prostheses placement and testosterone supplementation to maintain male sexual characteristic. Several types of testosterone compounds and various modes of hormone delivery are currently used clinically, however, their pharmacokinetic properties are not ideal. In this study we explored the possibility of creating hormone releasing testicular prostheses that could continuously supply and maintain physiologic levels of testosterone in vivo over time. Chondrocytes harvested from bovine articular cartilage were seeded on testicular shaped polymer scaffolds at a concentration of 100 x 10^6 /ml. Scaffolds were maintained in a bioreactor for 4 weeks to form cartilage tissue. Subsequently, testosterone enanthate (100 ug) was injected into the central hollow space of each testicular prosthesis. Some prostheses were observed in vitro, and another group was implanted into the scrotal space of castrated athymic mice (n=10). The implanted prostheses showed an initial burst effect of testosterone followed by a broad plateau for 16 weeks (> 500 ng/dl) and a decreased level of testosterone until 40 weeks. The testosterone levels were physiologic throughout the 40 weeks and the entire testosterone released was calculated as 60% of the injected volume. The circulating testosterone levels in the protheses implanted animals demonstrated a maximum peak on day one and a continued physiologic range during the entire study period. This study demonstrates that engineered cartilage testis can be created in bioreactors, can be implanted in vivo, and can release testosterone for a prolonged period. Furthermore, the levels of testosterone release can be maintained within the physiologic range. Periodic reinjection may potentially provide permanent physiologic hormonal replacement. This novel technology may be beneficial for injured soldiers who require testicular prostheses and chronic hormone supplementation.

1. INTRODUCTION

Bilateral anorchia, either congenital or acquired, often requires placement of testicular prostheses and testosterone supplementation to achieve anatomical and functional restoration (Adshead et al. 2001; Baumrucker 1956; Hazzard 1953; Incrocci et al. 1999) . Although implantation of testicular prostheses is not vital to patient's survival and daily activities, several studies have suggested that testicular implants lead to improved body image and personal satisfaction (Incrocci et al. 1999; Money; Sollod 1978).

Silicone testicular prostheses, consisting of a silicone shell filled with silicone gel, were used clinically for several decades until a moratorium was imposed in 1992 due to safety concerns related to gel leakage (Barrett et al. 1991; Doherty et al. 2000; John; Fordham 2003; Marshall 1986). In addition, implantation of these prostheses has been associated with problems such as infection, inflammation and extrusion (John; Fordham 2003; Lappe 1993). Although saline filled silicone prostheses are currently being used clinically, long-term safety and biocompatibility have not yet been determined.

Although commercially available testicular prostheses are able to provide anatomical tissue restoration, normal testicular function, such as production of testosterone can not be achieved. As such, male hormone supplementation is necessary for the initiation and maintenance of secondary sexual characteristics in patients with absent testicular tissue (Aynsley-Green et al. 1976; Bernasconi et al. 1992; Jockenhovel 2004). Several types of testosterone compounds and various hormone delivery methods are currently being used clinically, but their pharmacokinetic properties are not entirely satisfactory (Jockenhovel 2003, 2004; Jockenhovel et al. 1996). The ideal substance for hormone supplementation should be long-acting, able to maintain consistent levels of circulating testosterone, economical, and it should not cause untoward effects (Ebert et al. 2005; Jockenhovel 2004; Schubert et al. 2004).

The use of transdermal testosterone patches for this purpose has become popular due to the

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convenience of the method. However, the duration of action is relatively short and frequently requires an additional patch to maintain physiologic testosterone levels (Mazer; Shifren 2003; Wang et al. 2000). Moreover, dermatological problems are prevalent, which often require patients to seek other methods of testosterone delivery, such as oral or injection. Oral testosterone pills are the first line treatment and are administered daily. However, this method of administration is often associated with increased erythropoiesis (Jockenhovel 2004; Weil et al. 1980; Zachmann 1991). Intramuscular injection of testosterone enanthate and testosterone undecanate is commonly used in patients because of the prolonged duration of action associated with these forms of testosterone. However, this method is associated with untoward effects, such as gynecomastia, and frequent injections (3-4 weeks) are required (Jockenhovel 2003, 2004; Schubert et al. 2004).

We previously reported that autologous cartilage tissue can be engineered using chondrocytes seeded on biodegradable polymers (Amiel; Atala 1999; Amiel et al. 2001; Atala 1998; Atala et al. 1993; Yoo; Atala 2002; Yoo et al. 1999). The polymers can be configured to achieve any shape or size, depending on the specific needs. We demonstrated that the engineered autologous cartilage tissue can be used to treat vesicoureteral reflux and incontinence, as penile prostheses, and as biological urethral stents (Amiel et al. 2001; Kim et al. 2002; Yoo; Atala 2002; Yoo et al. 2000; Yoo et al. 1999). In this study, we examined the feasibility of using engineered cartilage tissue, with its associated elastic properties, for the creation of testicular prostheses. The use of auotologous cells would diminish the risk of inflammatory and immunological reactions. We investigated the possibility of creating hormone releasing testicular prostheses that could continuously supply and maintain physiologic levels of circulating testosterone in vivo over time.

2. MATERIALS AND METHODS

2.1 Polymers

Unwoven sheets of polyglycolic acid polymers (density 58 mg/ml) were designed and configured into testis shaped scaffolds with a central hollow space (0.4 cm in diameter and 1 cm in length). The polymers were composed of fibers of 15μm in diameter and an interfiber distance between 0 - 200 um with 95% porosity. The polymer scaffolds were designed to degrade via hydrolysis in 6-8 weeks. The resulting flexible scaffold was coated with a liquefied copolymer (poly-L-lactide-co-glycolide 50:50; Sigma; St. Louis, MO; 80 mg/ml methylene chloride) in order to achieve adequate mechanical strengths to maintain structural integrity. The polymers were sterilized in ethylene oxide and placed under sterile conditions until cell delivery.

2.2 Cell isolation, culture and seeding

Hyaline cartilage was obtained from the articular surfaces of calf shoulder. The shoulders were washed in Povidone-iodine 10% solution, and dissected into 2 to 3 mm tissue fragments. Chondrocytes were isolated under sterile conditions using a method reported previously (Yoo et al. 1999)Briefly, the cartilage tissue fragments were digested in Ham's F-12 culture medium containing 3% type II collagenase for 6 to 12 hours (Worthington Biochemical Corp., Lakewood, NJ). The recovered cells were rinsed in phosphate buffered saline (PBS) and re-suspended in culture medium (Ham's F-12 containing 10% FBS, penicillin 100 U/cm^3, and streptomycin 100 μg/cm^3, ascorbic acid 50 μg/ml; Gibco; Grand Island, NY). Cell number and viability were determined by trypan blue exclusion under light microscopy. Chondrocytes were plated and grown in culture at 37^{o}C in 5% CO_2 until a sufficient number of cells was obtained. Subsequently, the cells were trypsinized, collected, washed and counted for seeding.

2.3 Testicular Prosthesis: In vivo tissue formation

Chondrocytes were seeded onto pre-formed poly-L-lactic acid coated polyglycolic acid polymers at a concentration of 50 x 10^6 chondrocytes/cm^3. A total of 32 poly-L-lactic acid coated polyglycolic acid polymer scaffolds seeded with cells were implanted in the scrotal space of 16 athymic mice. A control group consisting of 16 polymer scaffolds without cells was implanted into the scrotal space of 8 animals. The animals were sacrificed at 1, 2, 3 and 6 months after implantation for analyses (n=12 samples per time point; 4 with cells and 2 without cells).

2.4 Testicular Prosthesis: In vitro functional studies

Chondrocytes were seeded onto 10 pre-configured testis shaped polymers at a concentration of 100 x 10^6 chondrocytes/cm^3. Five days after seeding, the cell-polymer constructs were suspended freely and maintained in a rotating flask bioreactor for 4 weeks to form cartilage tissue. Subsequently, testosterone enanthate (100 g) was injected into the central hollow space of each testicular prosthesis, and

these prostheses were maintained for 40 weeks in culture. A sample of medium was collected every 2 days and testosterone levels in the medium were measured.

2.5 Testicular Prosthesis: In vivo functional studies

In addition to the aforementioned experiments, a separate group of ex-vivo engineered testicular prostheses was prepared and implanted into the scrotal space of castrated athymic mice (n=10). Testosterone enanthate (100 g) was injected into each prosthesis. The control groups consisted of animals with castration only (n=8) and sham operations (n=5). Blood samples from every animal were collected prior to and 2 weeks after castration, 1 day after testosterone administration, and weekly up to 16 weeks for circulating testosterone level measurement. The engineered testicular prostheses were retrieved at sacrifice for analyses.

2.6 Histological and microscopic analyses

Five micron sections of formalin-fixed paraffin-embedded tissues were cut and stained with hematoxylin and eosin (H&E), aldehyde fuschin-alcian blue, safranin-O, toluidine blue and Masson's trichrome. In addition, scanning electron microscopy (SEM) was performed to determine the distribution and extent of cartilage tissue formed within the polymer scaffolds prior to and at 1, 2, 3 and 6 months after implantation. Specimens for SEM were fixed in 1% (v/v) buffered glutaraldehyde and 0.1% (v/v) buffered formaldehyde for 30 minutes and 24 hours, respectively. After dehydration with a graded ethanol series, the samples were air-dried. The dried samples were mounted on aluminum supports and sputter coated with gold. The samples were visualized using a scanning electron microscope (JOEL, model JSM-35, Peabody, MA) with a voltage of 25-kV.

2.7 Biomechanical Studies

Mechanical properties of the engineered testicular prostheses were assessed using a mechanical tester (model 5542, Instron corp., Canton, MA) with a 500 N-maximum load cell (n=3). The longitudinal axis of the specimens was compressed until it reached 80% of the initial thickness and released to its initial position at a cross-head speed of 0.5 in/min. The compressive modulus was obtained from the slope of the initial linear section of the stress-strain curve.

2.8 Collagen content assay

The total collagen content per unit dry weight of the engineered prosthesis samples was determined (n=3 per time point). Briefly, the retrieved tissue samples were lyophilized, homogenized and hydrolyzed (6N HCl at 130^{o} C for 3 hrs) to obtain tissue extracts. After neutralization with 2.5 N NaOH, hydroxyproline oxidation was initiated by adding 1 ml chloramine-T to the extract. After gentle mixing and incubation for 20 minutes, the chloramine-T was destroyed by addition of 1 ml perchloric acid to each tube. Finally, 1 ml p-dimethylamino-benzaldehyde solution was added and incubated for color development. The absorbance was measured using a spectrophotometer at 550 nm. The standard curve was plotted using a linear regression analysis.

2.9 Hormone Detection Assay

Testosterone levels, contained in the collected samples, were determined by radioimmunoassay (Lin et al. 2007; Miechi et al. 1975; Schalch et al. 1968) using the procedures supplied by the manufacturer followed by measuring the testosterone levels in an automatic gamma counter (Lbk Wallac, Monterey, CA).

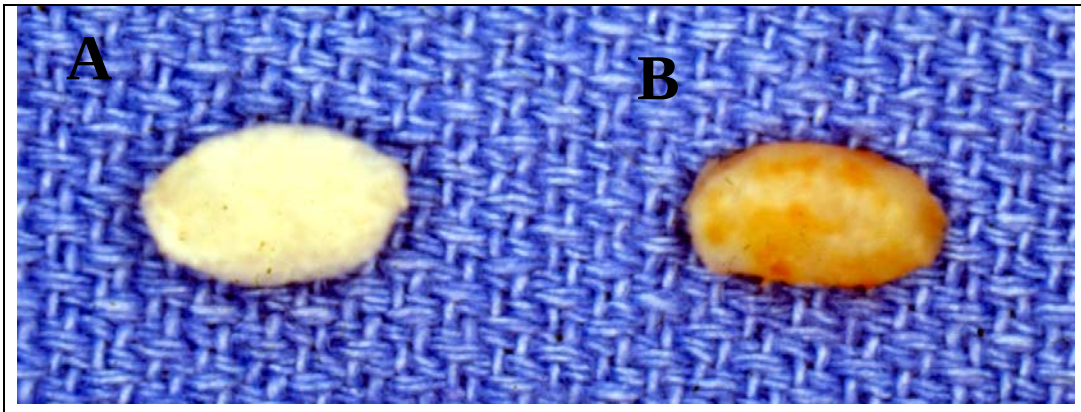

Figure 1. Gross examination. A) Pre-configured testicular prosthesis prior to cell seeding. B) Testicular prosthesis at retrieval showing well formed cartilage structure.

3. RESULTS

3.1 In vivo cartilage formation

All animals tolerated the implants for the entire duration of the study without any noticeable complications. Gross examination at retrieval showed the presence of well formed milky white cartilage structures within the scrotum (Figure 1). There was no evidence of erosion or infection in any of the implantation sites. The average wet and dry weight of the retrieved specimens increased gradually until 3 months, and remained constant at 6 months after implantation.

Compressive biomechanical analyses of the retrieved prostheses demonstrated that the engineered cartilaginous tissue was readily elastic and withstood high degrees of pressure. The compression modulus, obtained from the slope of the initial linear section of the stress-strain curve was 0.22, 0.49, 0.8, 1.47 and 1.67 kgf at 1, 2, 3 and 6 months, respectively. SEM of the pre-implanted cell polymer scaffolds demonstrated uniform cell attachment on the polymer fibers. The cartilage prostheses retrieved at 1 month after implantation demonstrated the formation of extracellular matrices, which occupied interfibrillar space within the polymer scaffolds. Undegraded polymer fibers were evident at 1 month. However, solid cartilage tissue structure was present by 3 months after implantation.

Histologically, all of the experimental specimens seeded with cells demonstrated mature chondrocytes accompanied by a basophilic extracellular tissue matrix at all time points (Figure 2). Presence of undegraded polymer fibers was evident at 1 month after implantation and these fibers continued to degrade over time. The control scaffolds without cells failed to show cartilage tissue formation at all time points. Aldehyde fuschin-alcian blue, toluidine blue, Safranin-O and Masson's trichrome staining further confirmed that the scaffolds contained cartilage tissue with normal composition and structure.

Collagen content assay demonstrated that the testicular prostheses seeded with cells had an average collagen composition fraction of 6.5 % and 10.8% of the total dry weight at 1 and 2 months, respectively. The collagen content then remained constant up to 6 months after implantation. Collagen was not detected in control implants without cells.

3.2 Testicular Prosthesis: In vitro functional studies

In the bioreactor studies, milky white cartilage was formed by 4 weeks on every testicular prosthesis. The ex-vivo prostheses, injected with 100 μg testosterone enanthate, produced an initial burst of testosterone in the

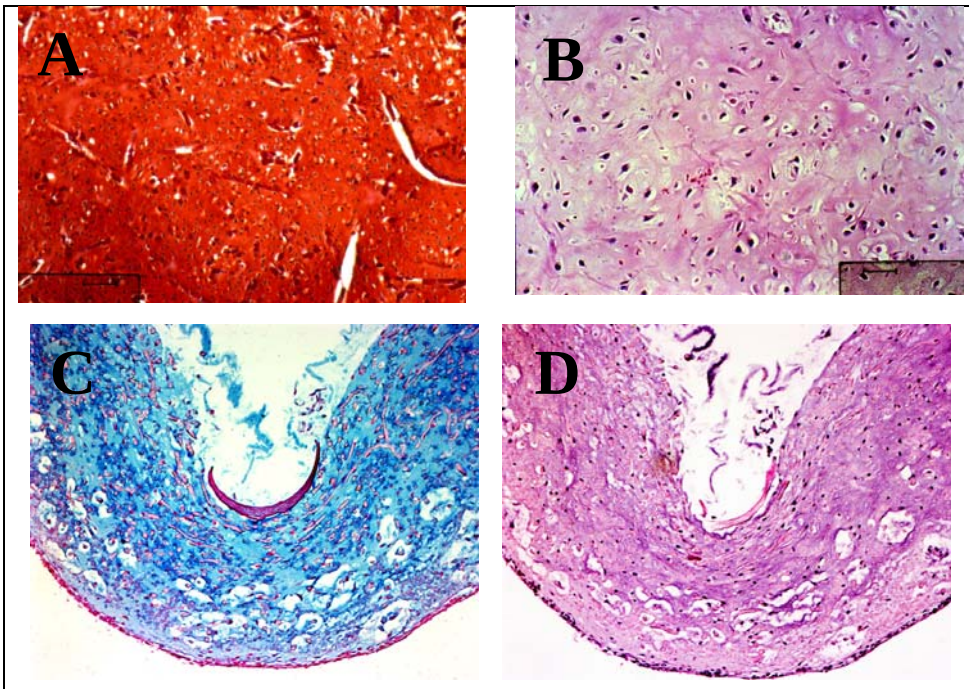

Figure 2. Testicular prostheses seeded with cells demonstrate mature chondrocytes accompanied by extracellular tissue matrices. A) In vivo formed prostheses. Presence of undegraded polymer fibers are observed at 1 month (Safranin-O;100x). B) Polymer fibers are entirely degraded at 6 months after implantation (H&E 250x). C, D) Retrieved ex vivo formed testicular prostheses at 3 months after implantation (C) Alcian blue; 40x , (D) H & E; 40x.

media, followed by a broad plateau for 16 weeks (> 500 ng/dl). After this, testosterone levels gradually decreased until 40 weeks. The detected testosterone levels were physiologic throughout the entire study period (40 weeks). The accumulated amount of the released testosterone was calculated as 60% of the initial injection concentration. Histological analyses of all constructs formed in the flask bioreactor demonstrated cartilaginous tissue structure composed of mature chondrocytes surrounded by extracellular matrices.

3.3 Testicular Prosthesis: In vivo functional studies

All animals tolerated the implants for the entire study period. There was no evidence of erosion or infection in any of the implanted animals. In the animals implanted with testosterone-containing prostheses, the circulating testosterone levels peaked on day 1 and then continued to maintain physiologic levels throughout the entire study period (16 weeks). The control animals that received a single intramuscular injection of testosterone demonstrated bursts in hormone levels during the first 3 weeks and then these levels decreased to castrate level by 7 weeks after administration (Figure 3). Histologically, the retrieved testicular implants were composed of an outer layer of mature chondrocytes with a hollow spacious center in each prosthesis (Figure 2).

4. DISCUSSION

The need for adequate reconstruction of both testes in patients with bilateral anorchia cannot be overemphasized, as it is critically associated with normal psychosexual development (Adshead et al. 2001; Aynsley-Green et al. 1976; Money; Sollod 1978). Although the FDA approved saline-filled silicone prostheses are known to be safer than previous versions (silicone gel-filled), the long-term outcome has not been verified (Turek et al. 2004). In addition to prosthetic implantation, testosterone supplementation is usually necessary to achieve and maintain secondary sexual characteristics (Jockenhovel 2003, 2004). In this study, we show that engineering of cartilaginous testicular prostheses using biodegradable polymers seeded with chondrocytes is possible (Amiel et al. 2001; Atala

1998; Atala et al. 1993). We also demonstrate that a large amount of testosterone can be loaded within the engineered tissue and then released over time. These findings demonstrate that a tissue-engineered, multi-functional testicular device is able to maintain physiologic levels of serum testosterone for 16 weeks when implanted in the scrotum of castrated animals.

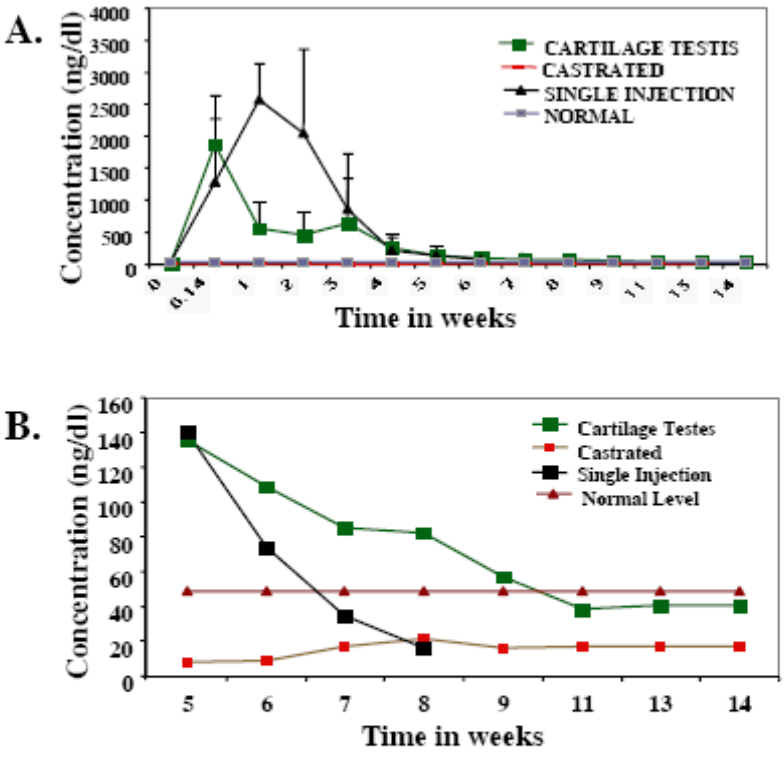

Figure 3. The circulating testosterone levels in the animals implanted with testosterone containing prostheses maintained continued physiologic levels during the entire study period. A) Entire graph. B) Enlarged graph.

The prosthetic tissue engineered with chondrocytes has several advantages over the synthetic saline-filled silicone prostheses. If this technology were used with autologous cells in a clinical setting, the engineered tissue would be biocompatible, non-immunogenic and non-antigenic, thus eliminating some of the problems associated with silicone implantation. This has been tested and confirmed repeatedly in many experimental and clinical studies using autologous cell-based applications (Amiel et al. 2001; Atala et al. 1994; Atala et al. 1993; Kim et al. 2002; Yoo; Atala 2000, 2002; Yoo et al. 1998). In this study we have demonstrated that none of the animals experienced adverse effects resulting from the engineered prosthesis, and all animals survived for the entire duration of the study. In addition, the engineered cartilaginous testis possessed the desired mechanical characteristics, including innate elasticity and resistance to compressive forces. Therefore, problems such as prosthesis rupture or erosion would be unlikely to occur in vivo.

The use of synthetic polymers as cell carriers and support matrices is attractive because they can degrade in a controlled manner (Yoo; Atala 2002; Yoo et al. 1999). Here, we used polymer scaffolds that are designed to degrade over a period of two to three months, which provided adequate time for tissue formation while maintaining structural integrity. The structural architecture of the flexible

polymer scaffolds was strengthened in this study by the addition of a liquefied copolymer (poly-L-lactide-co-glycolide 50:50), and, as the fibers were designed to degrade within 2 to 3 months, cartilage tissues consisting entirely of cellular components eventually formed. Moreover, maintenance of prosthetic tissue shape and size is required and largely depends on the structural configuration of the polymer scaffolds. As such, polymers with 95% porosity and adequate interfibrillar distance were used in this study to promote diffusion and to provide sufficient cell accommodation. As a result, these cartilage tissues engineered in vivo maintained their initial shape and size over time.

Cartilage tissue is best characterized as an avascular structure with abundant extracellular matrix(Yoo; Atala 2000; Yoo et al. 1999). The seeded chondrocytes readily adhered to the polymer fibers and progressively formed mature cartilage tissue in vitro and in vivo, replacing the degrading polymer fibers over time, as demonstrated by scanning electron microscopy. Adequate formation of mature cartilage was achieved, as evidenced by the presence of chondrocytes within lacunae and the presence of highly sulfated mucopolysaccharides. In this study extracellular matrix also formed within the engineered prosthesis and consisted mainly of collagen. The amount of collagen production progressively increased up to 2 months and then remained constant, which indicates the stability of the extracellular matrix.

In this study we show that the testosterone, placed in the central hollow space of the engineered prosthesis, was released over the entire study period (40 weeks ex vivo and 16 weeks in vivo). Although this study did not investigate the exact mechanism of testosterone release, we believe that the release was facilitated by passive diffusion, similar to the way in which mature chondrocytes obtain oxygen and nutrients (Amiel et al. 2001; Yoo et al. 1999). If this were correct, the release kinetics of testosterone would be affected by polymer design and configuration, cell seeding density, and initial testosterone concentration. However, confirmation of the release mechanism is critical for optimization of the control of testosterone release through the prosthetic wall over a target period.

In addition, we have shown that the engineered cartilaginous testicular prostheses are able to release testosterone over a longer period of time than a single intramuscular injection of testosterone enanthate, which only lasts up to 7 weeks. More importantly, the serum testosterone levels of the testosterone enanthate injection group were uncontrolled, with a rapid decrease in a linear fashion. These results are consistent with the clinical limitations of intramuscular injection of testosterone enanthate. These include frequent injections and an initial high testosterone bursting effect that can lead to gynecomastia (Schubert et al. 2004). In contrast, the engineered prostheses resulted in a lower initial testosterone bursting effect followed by maintenance of physiologic levels of serum testosterone.

5. CONCLUSIONS

This study clearly demonstrates that the engineered functional testicular prostheses offer advantages over the currently practiced therapies. The engineered tissue prostheses may augment the limitations and deficiencies of the current testosterone delivery methods. This technology could be used in patients who require testicular prostheses and testosterone supplementation. Autologous cartilage tissue could be obtained from a small biopsy in the outpatient clinic. Chondrocytes from this biopsy would be grown, expanded and seeded on pre-configured, computer generated three-dimensional polymer scaffolds. The testicular cartilage prostheses with an internal testosterone reservoir would be constructed ex vivo and then implanted into the same patient. This technology has the potential to become the preferred clinical management tool for anorchia and other disorders requiring testicular prosthesis application with testosterone supplementation. Further studies are currently being performed to optimize the testosterone release system for enhanced control, which is a necessary step for clinical application.

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